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Soil and Sediment Bacteria Capable of Aerobic Nitrate Respiration

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Several laboratory strains of gram-negative bacteria are known to be able to respire nitrate in the presence of oxygen, although the physiological advantage gained from this process is not entirely clear. The contribution that aerobic nitrate respiration makes to the environmental nitrogen cycle has not been studied. As a first step in addressing this question, a strategy which allows for the isolation of organisms capable of reducing nitrate to nitrite following aerobic growth has been developed. Twenty-nine such strains have been isolated from three soils and a freshwater sediment and shown to comprise members of three genera (*Pseudomonas*, *Aeromonas*, and *Moraxella*). All of these strains expressed a nitrate reductase with an active site located in the periplasmic compartment. Twenty-two of the strains showed significant rates of nitrate respiration in the presence of oxygen when assayed with physiological electron donors. Also isolated was one member of the gram-positive genus *Arthrobacter*, which was likewise able to respire nitrate in the presence of oxygen but appeared to express a different type of nitrate reductase. In the four environments studied, culturable bacteria capable of aerobic nitrate respiration were isolated in significant numbers (10^4 to 10^7 per g of soil or sediment) and in three cases were as abundant as, or more abundant than, culturable bacteria capable of denitrification. Thus, it seems likely that the corespiration of nitrate and oxygen may indeed make a significant contribution to the flux of nitrate to nitrite in the environment.

Nitrogen levels in the environment are affected by an interacting web of processes, including the oxidation of ammonium and nitrite (nitrification), the dissimilatory reduction of nitrate to ammonium (nitrate ammonification), and the dissimilatory reduction of nitrate via nitrite and gaseous nitrogen oxides to dinitrogen gas (denitrification) (12, 26, 28). Nitrogen may be lost from a system as the gaseous products of denitrification are released to the atmosphere. Pools of nitrate and ammonium can turn over every 24 h in a grassland soil (15). The leaching of nitrate from soil can lead to the loss of cations important for plant nutrition and to increases in soil and water acidity and rates of aluminum mobilization (1, 17, 43). Conversions between different ionic forms lead to variations in the rate of leaching of nitrogen from soils. Despite nitrate ammonification and denitrification being thought to be anoxic processes, nitrate respiration has been observed under aerobic conditions in a number of bacteria, including *Escherichia coli*, *Paracoccus denitrificans* GB17 (also known as *Thiosphaera pantotropha* [32]), and *Pseudomonas aeruginosa* (9, 16, 31, 40, 48).

A membrane-bound nitrate reductase with an active site in the cytoplasm is widespread among nitrate-respiring bacteria, including nitrate-ammonifying and -denitrifying species. This enzyme allows the oxidation of quinol by nitrate to be coupled to the generation of a transmembrane proton electrochemical gradient and thus has an important role in energy generation under anoxic conditions. Nitrate reductases located in the periplasmic compartment have also been described in *Rhodobacter capsulatus*, *Rhodobacter sphaeroides* f. sp. *denitrificans*, *Alcaligenes eutrophus*, *Paracoccus denitrificans* PD1222 and GB17, and *Pseudomonas putida* (5, 6, 8, 11, 34, 39, 42, 44, 50). Reduction of nitrate in the periplasm is not sensitive to the oxygen inhibition of nitrate transport across the cytoplasmic membrane that prevents reduction by the membrane-bound enzyme (2, 24). Consequently, the possession of a periplasmic nitrate reductase has been linked to the ability to respire nitrate in the presence of oxygen in laboratory strains. The oxidation of quinol by the periplasmic nitrate reductase is not thought to lead to the generation of a transmembrane proton electrochemical gradient but rather to act as a valve to help regulate redox balance and maintain the poise of the aerobic respiratory chain (11, 38, 40).

The periplasmic and membrane-bound nitrate reductases have distinct biochemical properties. Previously described periplasmic nitrate reductases are relatively insensitive to azide (at micromolar concentrations) and cannot reduce chlorate (5, 33). The membrane-bound dissimilatory nitrate reductase has been studied in several species but the best characterized is the enzyme from *E. coli* (EC 1.7.99.4) (46). Similar nitrate reductases have been shown to be involved in anaerobic nitrate reduction in *Paracoccus denitrificans*, *Pseudomonas aeruginosa*, *Pseudomonas denitrificans*, and *Pseudomonas stutzeri* (7, 10, 14, 25). Membrane-bound nitrate reductases also reduce chlorate to chlorite and are highly susceptible to inhibition by azide (5, 14, 35). The different abilities of nonphysiological electron donors to permeate the cell membrane may be exploited to differentiate between the two classes of nitrate reductase. Reduced methyl and benzyl viologen (MV^+ and BV^+, respectively) are able to act as electron donors to both enzymes in crude cell extracts. Intact cell membranes are permeable by BV^+ but relatively less permeable by MV^+. Therefore, in whole-cell assays, BV^+ acts as an electron donor to both membrane-bound and periplasmic nitrate reductases, whereas MV^+ donates electrons predominantly to the periplasmic enzyme (5, 27).

Bacteria capable of expressing membrane-bound nitrate reductases have been enumerated in a number of different environments (28). However, the importance of aerobic nitrate dissimilation in the environment has yet to be established. In pure culture studies with laboratory strains, aerobic nitrate respiration has been ascribed to the activity of the periplasmic nitrate reductase (5). Although periplasmic nitrate reductases have been identified in members of the α, β, and γ proteobac-

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TABLE 1. Physical characteristics of the soils and sediment

Sample site[a]	Texture	Particle size distribution (%)			Water content (%)	pH
		<2 μm	2 to 50 μm	50 to 2,000 μm		
S1	Sandy	1	2	97	6	4
S2	Coarse loam	8	55	37	18	6
S3	Fine loam	30	30	40	19	6
BB	ND[b]	ND	ND	ND	50	8

[a] S1, humo-ferric podzol; S2, brown earth; S3, gley; BB, Barton Broad sediment.
[b] ND, not determined.

teria, the numbers of bacteria capable of expressing this enzyme in different environments have not been determined. To address this question, an enrichment and selection procedure for bacteria expressing periplasmic nitrate reductases has now been developed. Previous studies have shown that aerobic growth on reduced carbon substrates such as butyrate produces an enhanced expression of the periplasmic nitrate reductase (37, 44). Thus, the initial enrichment was for isolates growing on butyrate, and those expressing a nitrate reductase were then identified by screening for nitrite accumulation.

MATERIALS AND METHODS

Sample sites. Samples were collected from three contrasting soils and from a freshwater sediment in autumn (Table 1). The three soils were the following: a humo-ferric podzol of the Red Lodge series (4, 45) under a *Pinus sylvestris* plantation at Brandon Park, Brandon, Suffolk, United Kingdom (grid reference TL 771851); a typical brown earth of the Sheringham series (4, 13), which had been harvested after cereal cultivation at Hole Farm, Hempstead, Norfolk, United Kingdom (grid reference TG 111355); and a gley of the Beccles series which had been under sugar beet cultivation at the Raveningham Estate, Norfolk, United Kingdom (grid reference TM 387954). Particle size distributions of these soils have been published previously (4, 45). Soil pH values were measured on site in a 1:1 soil-water slurry. Triplicate soil samples were collected from the upper 5 cm of each soil and stored in sealed plastic containers at 4°C for not more than 16 h before analysis. The freshwater sediment was collected from Barton Broad (grid reference TG 362215). Three replicate sediment cores were collected, and the upper 1 cm of sediment was extracted with a syringe. Samples were suspended by shaking in sterile 0.1% sodium cholate at 4°C for 30 min in a wrist-action shaker before dilution in the same solution.

Media and growth conditions. All bacterial growth was at 20°C either in shaken flask culture or on 9-cm petri dishes unless otherwise stated. Total culturable bacteria were counted after serial dilution onto nutrient agar containing cycloheximide (50 mg/ml) to inhibit fungal growth. Culturable anaerobic denitrifying bacteria were counted after plating serial dilutions onto the minimal medium of Harms et al. (22) with succinate (30 mM) as the sole carbon source and KNO_3 (10 mM), followed by anoxic incubation under pure nitrogen. Bacteria expressing a periplasmic nitrate reductase were isolated on solid minimal medium with butyrate (30 mM) as the sole carbon source and under oxic growth conditions. Colonies from the butyrate minimal medium were transferred to fresh plates, and after 2 days of incubation were tested for the ability to reduce nitrate to nitrite by the agar overlay technique of Glaser and DeMoss (21). Cultures for use in nitrate electrode studies were grown to the late exponential phase in 50 ml of LB medium (yeast extract [5 g/liter], tryptone [10 g/liter], NaCl [10 g/liter]) in 250-ml conical flasks shaken at 250 rpm.

Protein and chromosomal DNA extracts were made from cells grown in shaken culture in 5 ml of LB medium in 30-ml bottles and harvested during the late exponential phase. Assays using the electron donors MV^+ and BV^+ were carried out with whole cells grown to the late exponential phase on a semidefined medium (pH 7.5) containing K_2HPO_4 (2 g/liter), KH_2PO_4 (1 g/liter), NH_4Cl (1 g/liter), $MgSO_4 \cdot 7H_2O$ (0.3 g/liter), a 5-ml/liter concentration of a solution containing 4 mM $FeSO_4$, 5 mM citric acid, and 18 mM H_2SO_4, 1 ml of Vishniac trace element solution per liter (49), Casamino Acids (0.1 g/liter), yeast extract (0.1 g/liter), succinate (30 mM), and KNO_3 (10 mM). Denitrifying cultures were grown in 30-ml bottles filled with medium (pH 7.5) containing the following: K_2HPO_4 (0.6 g/liter), KH_2PO_4 (0.2 g/liter), $CaCl_2$ (0.1 g/liter), $MgSO_4 \cdot 7H_2O$ (0.5 g/liter), $(NH_4)_2SO_4$ (1.5 g/liter), yeast extract (3.0 g/liter), and KNO_3 (10 g/liter).

Classification and identification of isolates. For extraction of proteins, cells were suspended in a mixture of 0.1 M Tris-HCl (pH 6.75), 2% sodium dodecyl sulfate (SDS), and 0.1% mercaptoethanol and boiled for 5 min. Proteins were resolved on a discontinuous SDS-polyacrylamide gel (10% resolving gel) by the method of Laemmli (29) and stained with Coomassie blue. Tests for fluorescent pigment production, denitrification, arginine dihydrolase activity, starch hydrolysis, gelatin hydrolysis, and oxidase activity were carried out as described by Stolp and Gadkari (47). Chromosomal DNA was prepared from 1 ml of culture which was harvested by centrifugation, washed briefly with ice-cold acetone, and then resuspended in 100 μl of 0.25 M sucrose–50 mM Tris-HCl (pH 8.0). Cells were lysed at 37°C (for 10 min) with 15 μl of lysozyme (5 mg/ml); the addition of 75 μl of 20% SDS in Tris-EDTA followed. After the addition of 50 μl of 5 M NaCl, the lysate was placed on ice for 2 h. The lysate was centrifuged for 15 min, and the DNA was purified from the supernatant with the Magic Miniprep Kit (Promega) as described in the manufacturer's instructions. DNA coding for rRNA (16S rDNA) was amplified by the PCR with the primers pA and pH* described by Edwards et al. (18). Oligonucleotides pA and pH* correspond to coordinates 8 to 28 and 1542 to 1522 on the *E. coli* rRNA sequence. The products of the amplification were purified with a Centricon C-100 concentration tube and then used in cycle sequencing as described in the protocol supplied by Pharmacia. The fluorescently labelled oligonucleotides used to prime sequencing reactions were pC* and pD*, as described by Edwards et al. (18). The coordinates of these oligonucleotides on the *E. coli* sequence are 361 to 341 and 536 to 518, respectively. Sequences were aligned and phylogenetic trees were drawn by use of the CLUSTAL method of the program Megalign in the DNASTAR package. An alignment prepared with programs in the Genetics Computer Group package was subjected to bootstrap analysis, and trees were drawn with distance matrix methods by use of programs in the PHYLIP package (19).

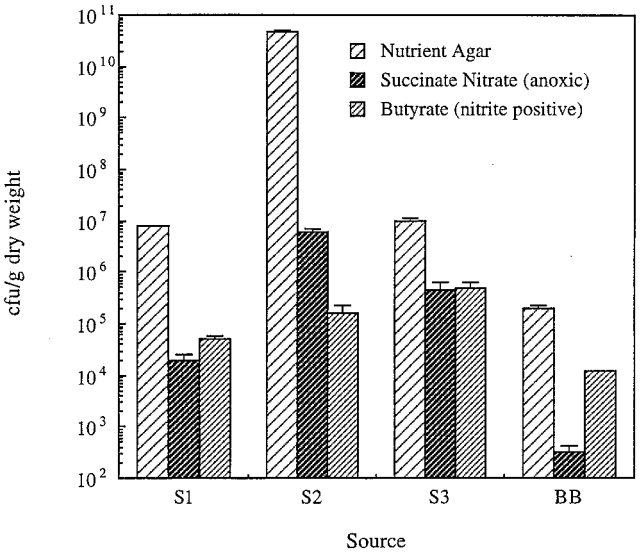

FIG. 1. Numbers of bacteria isolated from a humo-ferric podzol (S1), a brown earth (S2), a gley (S3), and the Barton Broad sediment (BB). Total viable counts were determined on nutrient agar, and denitrifying organisms were enumerated following anoxic incubation on succinate-nitrate minimal medium. Bacteria capable of accumulating nitrite in the agar overlay assay were enumerated following oxic incubation on butyrate minimal medium. Error bars are standard deviations.

Enzymology and nitrate electrode studies. Nitrate and chlorate reductase activities were measured in whole cells with either MV^+ or BV^+ as the electron donor (14). For in vivo measurements of nitrate reduction, sufficient cells to give an optical density at 600 nm of 0.6 were suspended in modified LB medium (containing no NaCl) in a 30-ml chamber fitted with a nitrate electrode (Orion) and an oxygen electrode (Jencons). Nitrate was added to a final concentration of 170 μM, and the cell suspension was stirred and sparged with air.

Nucleotide sequence accession numbers. The sequence data reported in this paper have been deposited in the EMBL database and assigned the accession numbers Z48263 to Z48291 and Z48428 for isolate S3.9.

RESULTS

Isolation and characterization of bacteria. The total numbers of culturable bacteria, culturable anaerobic denitrifiers, and bacteria capable of reducing nitrate to nitrite following aerobic growth on butyrate were determined for each site (Fig. 1). A total of 170 isolates capable of nitrate reduction after aerobic growth were selected for further work. Isolates were

TABLE 2. Physiological characteristics of bacteria isolated on the basis of their ability to reduce nitrate to nitrite following oxic growth on butyrate

Genus	Isolate	Fluorescent pigment production	Oxidase activity	Anaerobic arginine dihydrolase	Starch hydrolysis	Gelatin hydrolysis	Anaerobic denitrification
Aeromonas	BB3	−	+	+	+	+	−
	BB6	−	+	+	+	+	−
	BB7	−	+	+	+	−	−
	BB8	−	+	+	+	+	−
	BB16	−	+	+	+	+	−
	BB62	−	+	+	+	+	−
	BB66	−	+	+	+	+	−
	S3.33	−	+	+	+	+	−
Arthrobacter	S2.26	−	+	−	−	+	−
Moraxella	S2.18	−	−	−	−	−	−
Pseudomonas	BB49	−	+	+	−	−	−
	BB61	−	+	+	−	−	−
	S1.1	+	+	+	−	−	−
	S1.3	+	+	+	−	+	−
	S1.6	+	+	+	−	+	−
	S1.10	−	+	+	−	−	−
	S1.26	+	+	+	−	−	−
	S1.32	+	+	+	−	−	−
	S1.51	+	+	+	−	−	−
	S2.1	+	+	+	−	−	+
	S2.5	+	+	+	−	+	−
	S2.14	+	+	+	−	+	−
	S2.16	+	+	+	−	−	−
	S3.1	+	+	−	−	−	+
	S3.3	+	+	+	−	+	−
	S3.6	−	+	+	−	−	−
	S3.8	−	+	−	−	−	+
	S3.9	+	+	+	−	−	−
	S3.12	+	+	−	−	−	+
	S3.29	−	+	−	−	+	+

prefixed S1 (Red Lodge series soil), S2 (Sheringham series soil), S3 (Beccles series soil), and BB (Barton Broad sediment). The bacteria were grouped by their total cell protein profiles (data not presented) and physiological characteristics into 21 soil groups and 9 freshwater sediment groups (Table 2). Members of five groups (all pseudomonads) were able to grow anaerobically in the presence of nitrate. One isolate from each of the 30 groups was selected for 16S rDNA sequencing and further analysis. Three hundred nucleotides were sequenced from each amplified rDNA and used to generate a phylogenetic tree, allowing the assignment of each group to a particular genus (Fig. 2). The majority of strains (18 of 21) isolated from the three soils are members of the genus *Pseudomonas*, the remaining three being one member of each of the genera *Moraxella*, *Arthrobacter*, and *Aeromonas*. The majority of the isolates obtained from the Barton Broad sediment (seven of nine) were members of the genus *Aeromonas*, the remaining two being pseudomonads. To confirm the groupings and the accuracy of the amplification and sequencing methods, rDNAs from two isolates of the same group were sequenced for several groups, and the sequences obtained were shown to be identical.

MV^+- and BV^+-dependent nitrate reductase activities of isolates. Nitrate reductase assays were carried out on whole cells with the nonphysiological electron donors MV^+ and BV^+ (Tables 3 and 4). When grown in shaken flask culture with succinate as the carbon source, the ratios of BV^+-dependent nitrate reductase activity to MV^+-dependent nitrate reductase activity (BV^+/MV^+) were in the range of 3.3 to 0.4 for all of the isolates apart from *Arthrobacter* isolate S2.26, which showed a ratio of 6.5 (Table 3). In previous studies of bacteria

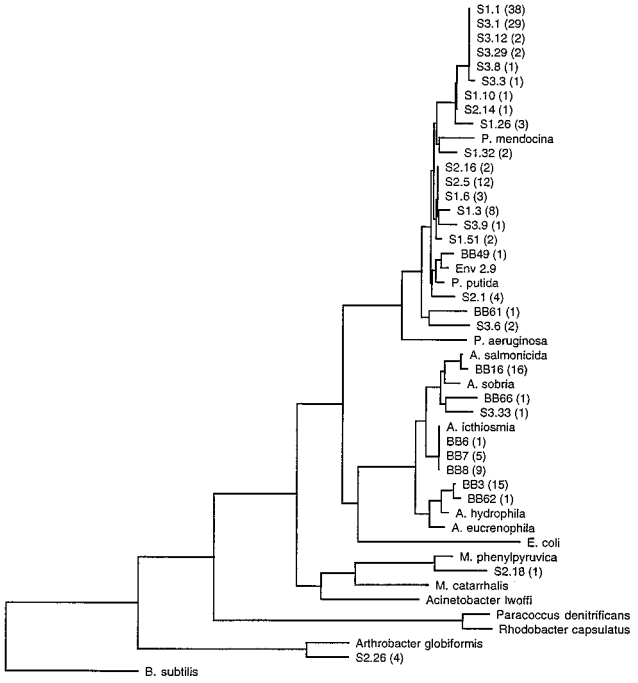

FIG. 2. Phylogenetic tree generated from 300 nucleotides of the rDNA sequences of the 30 isolates and a number of species deposited in the sequence databases. Env2.9 is a strain of *Pseudomonas putida* that has previously been shown to express a periplasmic nitrate reductase (11). Sequences were aligned and the tree was drawn by use of the CLUSTAL method of the Megalign program in the DNASTAR package. The numbers in parentheses indicate the numbers of individual isolates which were found in each group.

TABLE 3. Whole-cell nitrate reductase activities[a]

Genus	Isolate	MV^+-dependent nitrate reductase activity (nmol/min/mg)	BV^+-dependent nitrate reductase activity (nmol/min/mg)	BV^+/ MV^+ activity ratio	Azide inhibition (%)[b]	MV^+-dependent chlorate reductase activity (nmol/min/mg)
Aeromonas	BB3	60	40	0.7	0	60
	BB6	70	70	1	0	70
	BB7	200	250	1.3	0	220
	BB8	160	140	0.9	0	160
	BB16	110	40	0.4	0	0
	BB62	30	70	2.3	0	40
	BB66	40	40	1	0	30
	S3.33	160	280	1.8	0	0
Arthrobacter	S2.26	60	390	6.5	100	10
Moraxella	S2.18	50	70	1.4	80	0
Pseudomonas	BB49	30	70	2.3	0	20
	BB61	20	10	0.5	0	20
	S1.1	470	650	1.4	10	0
	S1.3	190	500	2.6	20	0
	S1.6	30	80	2.7	0	0
	S1.10	30	50	1.7	10	0
	S1.26	70	230	3.3	0	0
	S1.32	100	160	1.6	0	0
	S1.51	330	370	1.1	0	10
	S2.1	270	690	2.6	0	0
	S2.5	310	700	2.3	80	110
	S2.14	720	850	1.2	30	0
	S2.16	240	760	3.2	0	0
	S3.1	20	40	2	80	50
	S3.3	130	270	2.1	100	100
	S3.6	590	1,020	1.7	90	390
	S3.8	160	320	2	100	120
	S3.9	190	460	2.4	0	0
	S3.12	110	60	0.5	0	0
	S3.29	70	220	3.1	0	0

[a] Cultures were grown in shaken flasks with succinate as the carbon source. Nitrate reductase was measured with either MV^+ or BV^+ as the electron donor. Chlorate reductase activity was measured with MV^+ as the electron donor. All enzyme activities are nanomoles of electron donor oxidized per minute per milligram of protein.
[b] The sensitivity of the MV^+-dependent nitrate reductase activity to azide is expressed as percent inhibition.

expressing a membrane-bound nitrate reductase, BV^+-dependent activity was approximately sevenfold higher than MV^+-dependent activity, whereas the two rates were more closely matched (BV^+/MV^+ ratio, 0.9 to 1.7) in cells expressing a periplasmic nitrate reductase (5, 11). Therefore, the data obtained for the 29 isolates excluding *Arthrobacter* isolate S2.26 are consistent with the suggestion that these strains express a nitrate reductase with an active site external to the cytoplasmic membrane. When the isolates capable of growing anoxically by denitrification (pseudomonads S2.1, S3.1, S3.8, S3.12, and S3.29) were assayed after growth under these conditions, the ratios of BV^+/MV^+ were in the range of 6.6 to 153 (Table 4). A comparison of the BV^+/MV^+ ratios of these isolates grown oxically and anoxically indicates that they express an additional nitrate reductase during anaerobic growth which has its active site in the cytoplasm.

TABLE 4. Whole-cell nitrate reductase activities[a]

Pseudomonas isolate	MV^+-dependent nitrate reductase activity (nmol/min/mg)	BV^+-dependent nitrate reductase activity (nmol/min/mg)	BV^+/MV^+ activity ratio	Azide inhibition (%)[b]
S2.1	10	1,530	153	100
S3.1	50	330	6.6	100
S3.8	60	570	9.5	100
S3.12	30	260	8.7	100
S3.29	50	510	10.2	100

[a] Cultures were grown in standing vessels under denitrifying conditions. Enzyme activities were measured with either MV^+ or BV^+ as the electron donor, and units are nanomoles of electron donor oxidized per minute per milligram of protein.
[b] The sensitivity of the BV^+-dependent nitrate reductase activity to azide is expressed as percent inhibition.

Azide sensitivity and chlorate reductase activity. The sensitivity to azide was isolate dependent, with some isolates showing no inhibition of MV^+-dependent nitrate reductase activity at micromolar concentrations of azide but others showing complete inhibition. None of the *Aeromonas* isolates showed azide inhibition of MV^+-dependent nitrate reductase activity (Table 3). Both the *Arthrobacter* isolate S2.26 and *Moraxella* isolate S2.18 expressed azide-sensitive MV^+-dependent nitrate reductase activities. Among the *Pseudomonas* isolates, 11 showed no azide inhibition, 4 showed less than 30% inhibition, and 5 showed greater than 80% inhibition of MV^+-dependent nitrate reductase activity (Table 3). Half of the isolates showed no MV^+-dependent chlorate reductase activity while expressing MV^+-dependent nitrate reductase activity (Table 3). This provides further evidence that these isolates express a periplasmic nitrate reductase with no chlorate reductase activity. The remaining 15 isolates did express an MV^+-dependent chlorate reductase activity. Since chlorate is poorly permeable across the cytoplasmic membrane, the most likely explanation for this is that these isolates either express another periplasmic enzyme with chlorate reductase activity, for example a TMAO reductase (34), or that the periplasmic nitrate reductases of these isolates are capable of reducing chlorate.

TABLE 5. Nitrate reduction in vivo[a]

Genus	Isolate	Maximum dO_2 for nitrate reduction (% air saturation)	Nitrate reduction (nmol/min/mg)	Optimum dO_2 for nitrate reduction (% air saturation)	Nitrate reduction (nmol/min/mg)
Aeromonas	BB3	0	7	0	7
	BB6	80	10	80–0	10
	BB7	75	9	75–0	9
	BB8	80	10	80–0	10
	BB16	80	7	80–0	7
	BB62	0	5	0	5
	BB66	65	5	65–0	5
	S3.33	0	12	0	12
Arthrobacter	S2.26	80	42	80–0	42
Moraxella	S2.18	80	21	80–0	21
Pseudomonas	BB49	0	8	0	8
	BB61	0	5	0	5
	S1.1	80	41	80–0	41
	S1.3	80	29	80–0	29
	S1.6	80	9	80–0	9
	S1.10	80	17	80–0	17
	S1.26	80	15	80–0	15
	S1.32	80	27	80–0	27
	S1.51	80	11	80–0	11
	S2.1	80	12	80–0	12
	S2.5	80	13	80–0	13
	S2.14	50	50	10–0	74
	S2.16	80	20	80–0	20
	S3.1	0	21	0	21
	S3.3	80	13	80–0	13
	S3.6	80	71	80–0	71
	S3.8	80	7	0	24
	S3.9	20	46	20–0	46
	S3.12	80	18	80–0	18
	S3.29	0	12	0	12

[a] The maximum dissolved oxygen (dO_2) concentration (percent air saturation) which allowed nitrate respiration and the optimum oxygen concentration for nitrate respiration were measured for each isolate in a nitrate electrode. The rate of nitrate reduction (nanomoles of nitrate reduced per minute per milligram of protein) was measured at the maximum and optimum oxygen concentrations.

Nitrate respiration under oxic conditions. Nitrate reduction by aerobically grown whole cells was measured in a nitrate electrode over a range of oxygen concentrations. The electrode chamber was maintained initially at a dissolved oxygen concentration of 80% air saturation (~200 μM), and the rate of nitrate reduction was measured (Table 5). The dissolved oxygen concentration was then allowed to drop to 0% over 15 min, and the oxygen concentration at which the highest rate of nitrate reduction occurred was recorded (Table 5). The isolates showed differing responses to oxygen with respect to their ability to reduce nitrate. Nineteen of the isolates, including members of all of the genera isolated, were able to reduce nitrate at 80% air saturation. Of the remaining isolates, two (BB7 and BB66) were able to reduce nitrate at 75 to 65% air saturation. Two isolates (S3.9 and S2.14) were able to reduce nitrate at 20 to 10% air saturation, and the remaining seven isolates (BB3, BB62, S3.33, BB49, BB61, S3.1 and S3.29) were unable to reduce nitrate in the presence of oxygen (Table 5). In the case of those isolates able to reduce nitrate in the presence of oxygen, the rate of nitrate reduction was independent of the oxygen concentration, once reduction had begun, apart from *Pseudomonas* isolates S2.14 and 3.8. Isolate 2.14, although able to reduce nitrate at 50% air saturation, had a higher rate of nitrate reduction at 10% air saturation. Isolate S3.8 reduced nitrate at 80% air saturation but showed a threefold-higher rate of nitrate reduction in the absence of oxygen (Table 5). The addition of rotenone (5 μM), an NADH dehydrogenase inhibitor, to the electrode chamber completely inhibited the reduction of nitrate in all isolates apart from *Aeromonas* isolates S3.33, BB6, and BB7 and *Pseudomonas* isolate BB49. The *Pseudomonas* isolates S2.14 and S3.6, which had the highest rates of MV^+-dependent nitrate reductase activity, also exhibited the highest rates of nitrate reduction in the nitrate electrode. In all cases, the isolates respired oxygen in these experiments, indicating that when nitrate respiration was observed, it was occurring simultaneously with oxygen respiration.

DISCUSSION

This study has clearly demonstrated that bacteria that are capable of reducing nitrate to nitrite in the presence of oxygen may be isolated from a variety of habitats. The combination of enrichment for butyrate utilization and screening for nitrite accumulation identifies at a high frequency bacteria expressing a periplasmic type of nitrate reductase and allows for their enumeration. Of the 30 groups of organisms isolated, 23 showed significant rates of nitrate reduction in vivo in the presence of oxygen. This demonstrates that the periplasmic nitrate reductase provides the biochemical apparatus for aerobic nitrate respiration. In some cases, the periplasmic nitrate reductase was present, but nitrate respiration proceeded only in the absence of oxygen, indicating that other physiological factors may determine whether the process operates. Bacteria which are capable of expressing a nitrate reductase with an active site in the periplasm are clearly present in abundance in some environments. In two of the soils, the numbers of culturable bacteria with this capability were comparable to those of culturable anaerobic denitrifiers. In the freshwater sediment,

culturable bacteria expressing the periplasmic-type nitrate reductase were approximately 40-fold more abundant than culturable anaerobic denitrifiers. Thus, it is possible that oxygen-insensitive nitrate respiration may make a significant and previously unrecognized contribution to the flux from nitrate to nitrite in oxic and micro-oxic environments. The fact that only culturable organisms have been studied makes it likely that this approach underestimates the true numbers of organisms that are able to dissimilate nitrate under oxic conditions. Only three of the isolates (belonging to the genus *Arthrobacter*) appeared not to express the periplasmic-type nitrate reductase, which is perhaps not surprising given that these are gram-positive organisms.

Besides nitrate respiration, the production of nitrite detected in the screening procedure could, in principle, also be due to the assimilatory reduction of nitrate. This is regarded as unlikely since expression of the enzymes of nitrate assimilation is expected to be repressed by the presence of ammonium and nitrate and nitrite reductions are typically highly coupled during assimilation such that nitrite does not accumulate. The inhibition of nitrate reduction by rotenone (an inhibitor of NADH dehydrogenase) is similar to that found in electron transport chains to periplasmic nitrate reductases in *Pseudomonas putida* 2.9, *Paracoccus denitrificans*, *R. capsulatus*, and *R. sphaeroides* f. sp. *denitrificans* (11, 34, 39, 52). This confirms that nitrate reduction is a respiratory process in the environmental isolates and suggests that the organization of the electron transport chain which terminates in the periplasmic nitrate reductase is broadly similar in the laboratory strains and environmental isolates. The isolates in which nitrate reduction was not inhibited by rotenone might possess barriers to the entry of rotenone into the cell, for example, polysaccharide sheaths, or express rotenone-insensitive NADH dehydrogenases similar to those previously described (51).

The use of MV^+ and BV^+ has provided a useful tool for distinguishing between nitrate reductases with active sites in the periplasm and cytoplasm. This approach has an advantage over antibody-based methods in that it does not rely on the enzymes being biochemically similar. It is apparent that the cytoplasmic membranes of all of the bacteria isolated are relatively impermeable by MV^+, regardless of any differences in their phospholipid contents. This is consistent with previous studies which have shown that MV^+ does not cross the phospholipid membranes of *E. coli*, *Paracoccus denitrificans*, rat liver mitochondria, chloroplasts, and artificial phospholipid monolayers (3, 5, 20, 23, 27, 41). The nitrate reductases with MV^+-accessible periplasmic active sites that have been identified in the present study differed with respect to their azide sensitivities. This may imply that these enzymes belong to different biochemical classes or simply have subtly different active-site structures.

There has been considerable speculation concerning the physiological role and the ecological implications of the corespiration of nitrate and oxygen (30). Recently, a member of the genus *Comomonas* has been isolated from an upflow anaerobic filter (where conditions are not strictly anoxic) and is able to respire nitrate in the presence of oxygen. It was suggested that the corespiration of nitrate and oxygen might offer a physiological advantage in environments subjected to a fluctuating oxygen availability (36). A similar suggestion has been made following the isolation of a strain of *Pseudomonas putida* in which the expression of the periplasmic nitrate reductase (which provides the apparatus for aerobic nitrate respiration) is clearly activated by oxygen limitation (11). The balance of evidence suggests that nitrate can function as an auxiliary oxidant for the disposal of excess reducing equivalents or under conditions where the availability of oxygen in some way limits the cell's ability to achieve redox balance. In ecological terms, the implication of this is that the corespiration of oxygen and nitrate may be particularly important in environments which are rich in reduced carbon or subjected to a limiting or fluctuating oxygen availability. The nitrite produced as a consequence of nitrate reduction catalyzed by periplasmic nitrate reductases is presumably further reduced by nitrite-respiring bacteria or used as a substrate by nitrite-oxidizing organisms. If the latter is the case, then estimations of nitrification rates based on measurements of nitrate could be lower than the true rate of nitrification (26).

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